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Short Communication

Bioreduction and precipitation of uranium in ionic liquid aqueous solution by *Clostridium* sp.

C. Zhang [a,b,*], C.J. Dodge [c], S.V. Malhotra [a,1], A.J. Francis [c,d]

[a] Department of Chemistry and Environmental Science, New Jersey Institute of Technology, New York, NJ 07104, United States
[b] College of Environmental Science and Engineering, Nankai University, Tianjin 300071, China
[c] Environmental Sciences Department, Brookhaven National Laboratory, Upton, NY 11973, United States
[d] Division of Advanced Nuclear Engineering, Pohang University of Science and Technology, Pohang 790-784, South Korea

HIGHLIGHTS

- Uranium forms various complexes with ionic liquids.
- Uranium bioreduction was affected by the type of complex formed with ionic liquid.
- Precipitation of reduced uranium was retarded in the presence of certain ionic liquid.

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ABSTRACT

The ionic liquids, 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6], *N*-ethylpyridinium-trifluoroacetate [EtPy][CF_3COO] and *N*-ethylpyridiniumtetrafluoroborate [EtPy][BF_4], affected the reduction and precipitation of uranium by *Clostridium* sp. to a varying degree. Characterization of uranium association with the ionic liquids showed that uranium formed a monodentate complex with the anion BF_4^- and PF_6^- of [EtPy][BF_4] and [BMIM][PF_6], respectively; and a bidentate complex with carboxylate of [EtPy][CF_3COO]. Bioreduction of U(VI) was influenced by the type of complex formed: monodentate complexes were readily reduced whereas the bidentate complex of U(VI) with [CF_3COO] was recalcitrant. [EtPy][BF_4] affected the rate and extent of precipitation of the reduced uranium; at higher concentration the reduced U(IV) remained in the solution phase. The results suggest that by tuning the properties of ionic liquids they may be valuable candidates for uranium biotreatment.

1. Introduction

Contamination of soil, sediment and groundwater with uranium from mining and milling operations, radioactive wastes, and from nuclear accidents is a major environmental problem (Handley-Sidhu et al., 2010). Various chemical and biological methods such as ion-exchange (Li and Zhang, 2012), biosorption (Cecal et al., 2012), and bioreduction (Gao and Francis, 2008) have been reported to successfully remove or precipitate uranium. Among them bioreduction has been considered as an environmentally friendly and greener alternative for cleanup of uranium contamination. U(VI) are highly soluble and so are relatively mobile and biologically available in the environment. Under reducing conditions (e.g. subsurface) the transformation of U(VI) to sparingly soluble U(IV) species would significantly decrease its mobility and bioavailability.

Ionic liquids (ILs) have a number of potential applications in nuclear industry (Rout et al., 2012). An important consideration is their solvent stability. Allen et al. (2002) demonstrated that ILs are significantly more stable than mixtures of tri-*n*-butylphosphate and kerosene which is widely used solvent systems in the PUREX process for the separations of actinides from spent fuel. Shkrob et al. (2007) showed that ILs could protect the extractant tributylphosphate from radiolytic damage.

Meanwhile, ILs are biocompatible and may be of particular interest in the biocatalysis. As a novel medium, they offer many advantages including better substrate dissolution, improved enzyme thermal stability, enhanced enzyme selectivity, and more synthetic strategies (van Rantwijk and Sheldon, 2007). The interaction of ILs with uranium as means of extraction and concentration from wastes has been pursued; however, no research has been done on the microbial transformation of uranium complexed with ILs. In this study we investigated the effects of ILs on bioreduction of uranium by *Clostridium* sp. under anaerobic conditions. Under-

* Corresponding author at: College of Environmental Science and Engineering, Nankai University, Tianjin 300071, China. Tel./fax: +86 22 66229517.
E-mail addresses: zhangchengdong@nankai.edu.cn, zhangcd_nk@hotmail.com (C. Zhang).
[1] Current address: National Cancer Institute, Frederick, MD 21702, United States.

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standing the mechanisms of microbial catalyzed transformation of radionuclides in the presence of ILs may provide a potential strategy for selection of ILs for new applications, not only in separation chemistry but also in the biotreatment of radioactive wastes.

2. Methods

2.1. Chemicals

1-Butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6], *N*-ethylpyridiniumtrifluoroacetate [EtPy][CF_3COO] and *N*-ethylpyridiniumtetrafluoroborate [EtPy][BF_4], were synthesized and purified according to Zhao et al. (2003). The ILs were >98% pure as tested by NMR and FTIR. Uranyl nitrate ($UO_2(NO_3)_2{\cdot}6H_2O$) was obtained from BDH Chemicals, Analar, Poole, England.

2.2. Bacterial culture and growth medium

Clostridium sp. (ATCC 53464), isolated from coal cleaning waste, is a Gram positive, N_2-fixing, strictly anaerobic fermentative bacterium capable of reduction of U(VI) to U(IV) (Francis and Dodge, 2008). The bacterium was grown in a mineral salts (MS) medium (composition see support information) and the pH was adjusted to 6.5. The medium was pre-reduced by boiling and purging with N_2 gas for 15 min to remove dissolved oxygen. The medium was then allowed to cool and transferred to an anaerobic glove box filled with 95% N_2 and 5% H_2. Forty milliliters of the medium was dispensed into a 60 mL serum bottle. The bottle was then sealed by a butyl rubber stopper with an aluminum cap and autoclaved.

2.3. Preparation of uranium–IL complex

Uranyl ion stock solution was prepared by dissolving uranyl nitrate ($UO_2(NO_3)_2{\cdot}6H_2O$) in deionized water. Equimolar amounts of U(VI)–IL was prepared by continuously mixing 1:1 of uranyl nitrate and IL solution in ultra-pure deionized water (Milli-Q plus, Millipore) in a volumetric flask. The mixture was allowed to equilibrate for 24 h in the dark to avoid photodegradation.

2.4. Extended X-ray absorption fine structure analysis (EXAFS)

The 1:1 U–IL mixtures (20 mM) was placed in heat-sealed polyethylene bags (0.2 mL). The bags were then mounted on a plastic sample holder and fluorescence measurements were obtained at the U L_{III} absorption edge (17.166 keV) on beam line X-11B at the National Synchrotron Light Source at Brookhaven National Laboratory New York, using a Lytle detector. Multiple scans (up to 5) were performed for each sample and the results averaged to minimize the signal to noise ratio.

The data were processed using a multi-step procedure including background subtraction and normalization to the edge jump height, followed by Fourier transformation of the k^3-weighted (2–12 $Å^{-1}$) spectra. The theoretical EXAFS modeling code FEFF6 was used to calculate the back-scattering phase and the amplitude information for the individual neighboring atoms. The amplitude reduction factor (S_{02}) was fixed at 1.0 for all the fits. The *r* factor was evaluated for goodness of fit.

2.5. Bioreduction of U–IL complex

Uranium–IL complex was prepared by mixing 75 μL of 140 mM U(VI) with 0.4 mL of [BMIM][PF_6], or [EtPy][BF_4], or [EtPy][CF_3COO] respectively and diluted with pre-reduced de-ion water to 3 mL. The mixture was kept in the dark overnight to equilibrate and then the aliquot was added to the 18 h old growing culture of *Clostridium* sp. in MS medium. The final concentration of U(VI) was 0.27 mM and ILs were 48 mM for [BMIM][PF_6], 45 mM for [EtPy][BF_4] and 54 mM for [EtPy][CF_3COO] in culture medium. In order to determine the effect of different concentration of [EtPy][BF_4] on bioreduction, U(VI) concentration was maintained at 0.27 mM and [EtPy][BF_4] concentrations in solution were changed to 4.5, 22.5 and 45 mM, respectively.

Two sets for each IL were prepared and one set was used for kinetic study and the other set was kept intact for 48 h for the uranium distribution analysis. For the kinetic study, an aliquot was withdrawn periodically and the sample was filtered through a 0.45 μm membrane filter and the concentrations of U(IV) and U(VI) in solution were determined according to the method detailed below. For uranium distribution analysis, the sample was centrifuged at 5000*g* for 20 min and the concentrations of U(VI) and U(IV) in solution were determined. The precipitate along with the cells was washed three times with 20 mM KCl. The uranium in the precipitate was then extracted with 10 mL of 5 mM citric acid. The concentrations of both reduced U(IV) and unreduced U(VI) in citrate extracts were determined as below. All experimental procedures were performed under anaerobic condition in a glove box.

2.6. Determination of U(IV) and U(VI) concentration

The U(IV) was quantified immediately by a colorimetric method based on the capacity of U(IV) to reduce Fe^{3+} at pH 3.5 in a solution containing excess Fe^{3+} (Vazquez et al., 2009). Total uranium was determined with the kinetic phosphorescence analyzer (Chemcheck, WA) after completely re-oxidizing the bioreduced U(IV). U(VI) concentration was calculated by subtracting U(IV) from total uranium concentration.

3. Results and discussion

3.1. Characterization structures of U–IL complex by EXAFS

Fig. S1 shows the k^3-weighted (2–12 $Å^{-1}$) raw EXAFS spectrum (A) and the Fourier transform (B) for the various U–IL mixtures. The fitting parameters are presented in Table 1. The uranyl nitrate consists of 2 axial oxygens at 1.77 ± 0.01 Å, and 4.5 ± 1.5 O_{eq} at 2.33 ± 0.01 Å. These parameters are consistent with previously determined values (Kelly et al., 2002) and indicate the uranyl nitrate is present in solution as hydrated form.

In the U:[EtPy][BF4] mixture, 3.5 ± 1.3 equatorial oxygen were found at 2.45 ± 0.02 Å. In addition, the fit includes 1.4 ± 0.4 fluoride atoms at 2.22 ± 0.01 Å, which suggests the presence of an inner-sphere monodentate U–F complex. Similar results were obtained by Gaillard et al.(2005), where for $UO_2BF_4^+$ complex, U–Q_{eq} was at 2.45 Å and the U–F bond length was present at 2.24 ± 0.02 Å. The presence of a hydrogen bond between a fluorine atom and the UO_2 associated water has been proposed based upon energy minimization computational studies (Gaillard et al., 2005).

In U:[EtPy][CF3COO] complex 2 O_{ax} were found at 1.77 ± 0.03 Å with 4.6 ± 2.5 O_{eq} atoms at 2.40 ± 0.03 Å, and 1.4 ± 0.8 C atoms at 2.92 ± 0.02 Å. The U–C length was similar to that found in a uranyl acetate complex (1.3 C at 2.91 Å) (Jiang et al., 2002). The distances usually observed for carboxylate O_{eq} atoms which form a bidentate bond with the uranyl ion is in the range of 2.40–2.50 Å. Hence, we propose a bidentate structure for this complex. The appearance of OH in the inner-sphere is evidenced by potentiometric titration (Fig. S2) and electrospray ionization-mass spectrometry analysis (Fig. S3). There were no obvious U–U interactions observed between 3.7 and 4.2 Å for either the U:[EtPy][BF4] or U:[EtPy][CF3-COO] complexes indicating they are present as mononuclear

Table 1
EXAFS structural parameters for uranium and U:IL mixtures.

Sample	pH	Atom	*N*	*R* (Å)	σ^2	*r* Factor
Uranyl nitrate (aq)	1.66	$U–O_{ax}$	2	1.77 ± 0.01	0.002 ± 0.001	0.11
		$U–O_{eq}$	4.5 ± 1.5	2.33 ± 0.01	0.006 ± 0.002	
1:1 U:[BMIM][PF_6]	1.54	$U–O_{ax}$	2	1.75 ± 0.01	0.001 ± 0.001	0.06
		$U–O_{eq}$	4.9 ± 1.4	2.35 ± 0.01	0.005 ± 0.001	
1:1 U:[EtPy][BF_4]	1.37	$U–O_{ax}$	2	1.76 ± 0.01	0.002 ± 0.001	0.08
		$U–O_{eq}$	3.5 ± 1.3	2.45 ± 0.02	0.005 ± 0.002	
		U–F	1.4 ± 0.4	2.22 ± 0.01	0.004 ± 0.002	
1:1 U:[EtPy][CF_3COO]	1.24	$U–O_{ax}$	2	1.77 ± 0.03	0.001 ± 0.001	0.03
		$U–O_{eq}$	4.6 ± 2.5	2.40 ± 0.03	0.014 ± 0.003	
		U–C	1.4 ± 0.8	2.92 ± 0.02	0.004 ± 0.007	

(*N*) coordination number, (*R*) interatomic distance, (σ^2) disorder parameter, and (*r*) reliability factor. The O_{ax} atoms were fixed at 2 in all fits.

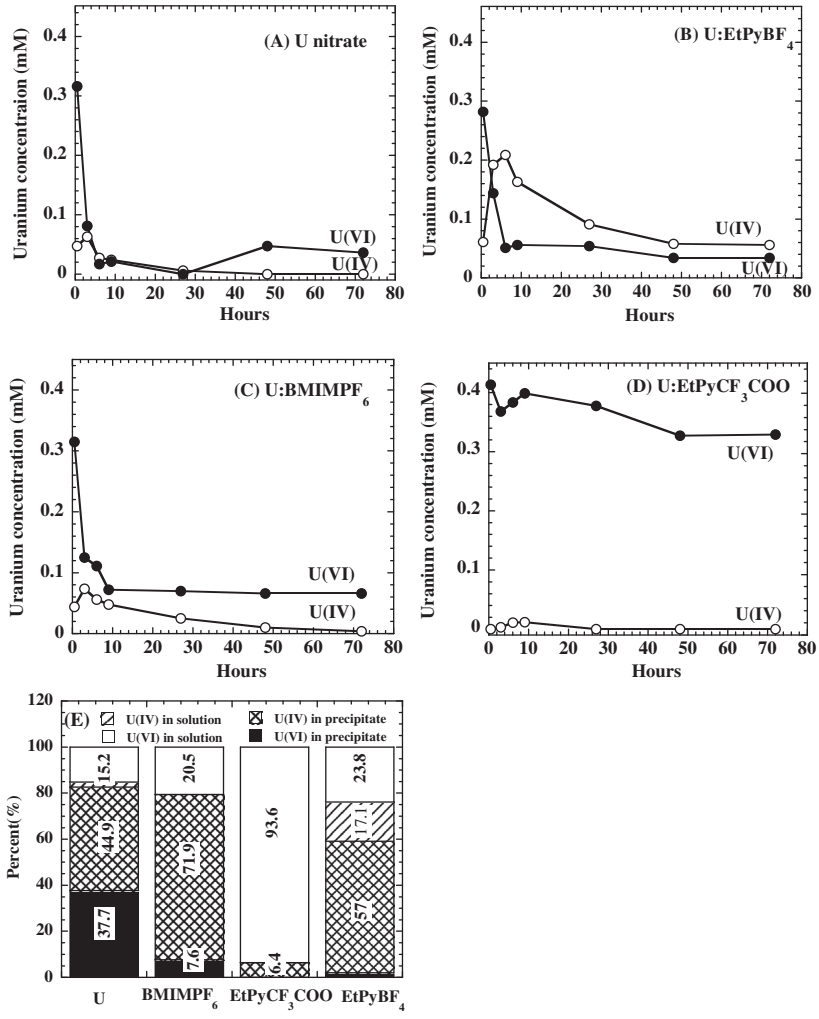

Fig. 1. Kinetic changes of uranium concentrations in solution during bioreduction in the presence of various ILs (A) without IL (control); (B) with [EtPy][BF_4]; (C) with [$BMIMPF_6$]; (D) with [EtPy][CF_3COO]; and (E) uranium distribution in the systems after bioreduction for 72 h.

form. The data confirm the formation of complexes between U and the anions of both [EtPy][BF4] and [EtPy][CF3COO].

EXAFS spectrum for the U:[BMIM][PF6] mixture does not indicate complexation of fluoride with uranyl ion but was similar to the hydrated uranyl nitrate form, which may be due to the low aqueous solubility of [BMIM][PF_6]. However, Gaillard et al. (2005) used time-resolved emission spectroscopy and computational studies to propose formation of a monodentate complex involving association of uranium with F^- and indicated the presence of a H bond with fluorine.

The complex formation between U and three ILs were also evidenced by potentiometric titration (Fig. S2), electrospray ionization-mass spectrometry (Figs. S3 and S4) and UV–vis spectrophotometry (Fig. S5). The structures of U–IL complex are proposed in Fig. S6.

3.2. Influence of ILs on uranium bioreduction

ILs affected to varying degree the bioreduction of U(VI) to U(IV) and its precipitation by *Clostridium* sp. In samples without ILs, the

U(VI) concentration in aqueous solution decreased from 0.33 to 0.02 mM in 8 h, then slightly increased to 0.04 mM at the end of experiment (Fig. 1A). The U(IV) concentration in solution phase showed a slight increase from 0.04 to 0.06 mM and it decreased to below detection level afterward. In the presence of [EtPy][BF_4] kinetic changes of U(VI) concentration in solution followed a similar trend as samples which received no ILs (Fig. 1B). However, the U(IV) concentration rose from an initial 0.06 mM to 0.22 mM in 8 h, and then it decreased at a rate of 0.04 mM h^{-1} to the final concentration of 0.06 mM. The results indicate the possible formation of U(IV)–BF_4 complex thereby preventing its precipitation. Reduction of uranium in the presence of [BMIM][PF_6] followed similar trend as uranyl nitrate (Fig. 1C). However, in the presence of [EtPy][CF_3COO] with which U(VI) formed a bidentate complex, U(VI) was not reduced by the bacterium and 94% of U(VI) was in solution after 72 h incubation (Fig. 1D).

The uranium distribution analysis (Fig. 1E) shows that after 72 h incubation the reduction efficiency decrease in the order of 74% for U-[EtPy][BF_4], 72% for U-[BMIM][PF_6], 47% for U-nitrate and 6% for U-[EtPy][CF_3COO]. It is interesting to notice that in U-[EtPy][BF_4] solution 17% of uranium was maintained in solution as U(IV). The results implied that the preferred reaction of U(IV) appeared to be recomplexation with BF_4^- ligand rather than precipitation as UO_2. Similar phenomena were also observed in other studies, where reduced uranium was present in solution as mononuclear U(IV)–citrate complex (Francis, 2006) or U(IV)–EDTA complex (Luo and Gu, 2011). This presumption was further evidenced by increasing of U(IV) concentration in solution in accordance with [EtPy][BF_4] concentration.

The bioreduced uranium in precipitate was confirmed by X-ray absorption near edge structure analysis (Fig. S7A) and UV–vis spectrometry analysis (Fig. S7B).

3.3. Effect of [EtPy][BF_4] concentration on uranium bioreduction

The effects of various concentration of [EtPy][BF_4] on uranium bioreduction are shown in Fig. 2. By increasing the [EtPy][BF_4] concentration, the maximum U(IV) concentration in the solution

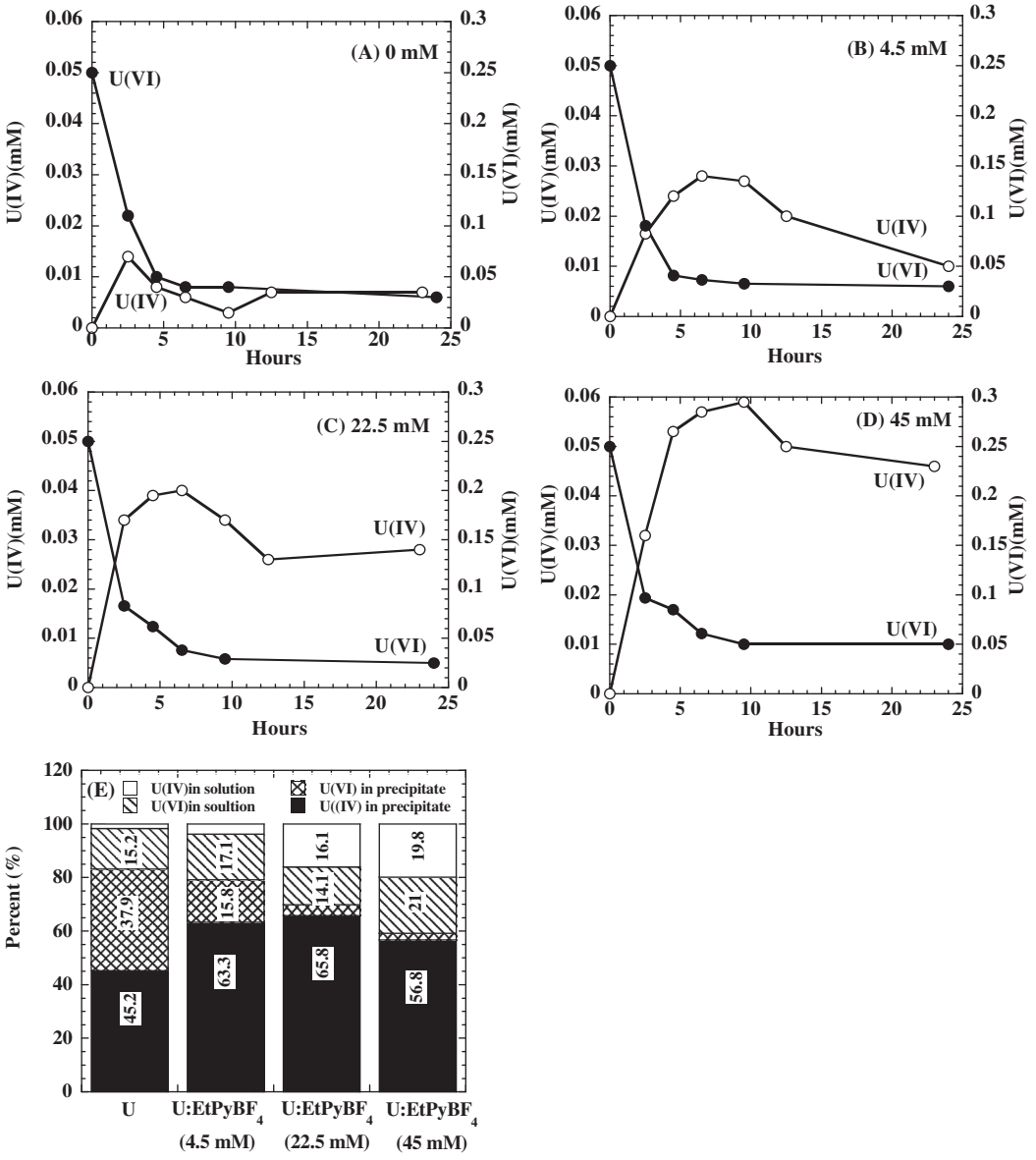

Fig. 2. Kinetic changes of uranium concentrations in solution during bioreduction in the presence of various concentration of [EtPy][BF_4] (A) 0 mM; (B) 4.5 mM; (C) 22.5 mM; and (D) 45 mM; and (E) uranium distribution in the systems after bioreduction for 72 h.

increased from 0.014 to 0.06 mM. Meanwhile, the precipitation of reduced U(IV) became much slower. The U(IV) in the solution phase increased in the order of 1.7%, 3.8%, 16.1% and 19.8% with the addition of 0, 4.5, 22.5, 45 mM of [EtPy][BF_4], respectively (Fig. 2E). The maximum reduction efficiency was observed with the addition of 22.5 mM of [EtPy][BF_4] which reached 82% (Fig. 2E). Therefore, in the presence of U–[EtPy][BF_4] complex the bioreduction extent was significant enhanced. Meanwhile, the remobilization of U(IV) could become an environmental concern. The U(IV) colloid formation in the presence of phthalate (Vazquez et al., 2009), humic acid (Gu et al., 2005) or other environmental matters have been reported to facilitate the transport of uraninite. Nevertheless, the stability of colloidal systems could be affected by many factors, such as electrostatic repulsion, steric hindrance, and solvation forces. The precipitation of uraninite would be feasible by proper tuning of ILs properties. In particular, we have demonstrated that [EtPy][BF_4] could be biodegraded under environmental conditions (Zhang et al., 2010). Thereby in the presence of other microorganism in the environment, the stability of uraninite will be changed.

The effect of ligands on bioreduction varied and the mechanism is still not clear. Two mechanisms for enhanced bioreduction have been proposed: (1) the ligands facilitate the electron transfer reaction by changing the speciation of the reactants and/or products or (2) facilitate bacterial metabolism and consequently increase U(VI) reduction efficiency (Boyanov et al., 2011). It is difficult to identify the intrinsic mechanism based on current data, future studies on the speciation-bioavailability relationship and genetic changes related to activity of the reductase will provide insightful information.

4. Conclusion

ILs could affect uranium bioreduction and precipitation by forming various complexes in aqueous solution. Future research on recyclability, toxicity and stability of ILs during biotreatment may provide useful information on their industrial application in uranium bioremediation. In general, ILs may not only be useful in the extraction of uranium, but also show high potential in the bioremediation of uranium waste stream.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.03.085.

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